Improvisations in phototrophy. Protein engineering and functional investigation of rhodopsin proton-pumps

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Chapter 6

General discussion and future outlook

In this project, we have made significant progress towards the spectral engineering and characterization of the proton-pumps proteorhodopsin from a SAR 86 γ-proteobacterium (PR) and *Gloeobacter violaceus* rhodopsin (GR). A combinatory strategy of selected protein mutagenesis with chromophore modification was utilized, which yielded several exciting results. We conceived the novel retinal analog MMAR, which has the remarkable potential, particularly when combined with specific opsin modifications, to generate NIR active proton-pumps. In order to screen for and/or optimize red-shifting protein mutants, we developed a novel chemotaxis-based directed evolution assay. This enables the simultaneous screening for desired spectral properties and pump function in a library of mutants. This assay can additionally be combined with retinal analogs, to enhance the proton-pumping function of the respective pigments. The stability of the various pigments generated in our study was assessed in different membrane-mimicking micro-environments, towards their eventual biophysical characterization. We anticipate that the library of retinal analog and rhodopsin proton-pump variants developed in this research will have broad potential in several biotechnological fields. Shifting the action spectra of microbial rhodopsins beyond 700 nm generates exciting applications in phototrophy, optogenetics, membrane-sensor- and nano-bio technologies.

6.1 Characterization and investigation of MMAR pigments

Alterations in the structure and electronic properties of the retinal chromophore have an impact on both the colour and pump activity of the resulting pigments, as demonstrated in **Chapters 2 and 3** of this thesis. Of the retinal analogs tested in **Chapter 2**, retinal A2 was found to induce a red-shift in the absorbance band of all pigments tested, while retaining significant proton-pump activity. Hence we further adapted A2 and tested various A2 analogs in **Chapter3**, of which MMAR was established as the most lucrative. All MMAR pigments showed red-shifted absorbance bands and could be activated by NIR light, thereby representing the first NIR active proton-pumps reported thus far. The properties of these MMAR pigments, particularly within the context of PR, are fascinating. The combination of PR-DNFS with MMAR results in an unprecedented ~740 nm absorbance band. In fact, this band is already intensified in WT PR upon protonation of the counterion. The unusual pH dependence of both PR:MMAR and PR-DNFS:MMAR illustrates the remarkable sensitivity of this analog towards the electrostatic distribution within the retinal binding pocket. We postulate that in MMAR pigments, the positive charge on the pSB has a tendency to delocalize towards the ring element. The 3-monoamine nitrogen provides a stabilizing influence to this charge leading to the resonance structures displayed in figure 3. 7. These structures probably exist in a relatively slow dynamic equilibrium, giving rise to the complex absorbance profile of the resulting pigments. The features of the electronic transitions involved in shaping this absorbance band, the influence of specific binding pocket environments towards the same, and their combined effects on the proton pump activity, provides inspiration for additional experimental and theoretical investigation.

Computational studies using time-dependent density functional theory (TD-DFT) and *ab initio* molecular dynamics (MD) have previously been very useful to simulate the potential energy surfaces involved in the ground

and excited states of the pSB and its photoisomerization dynamics [1, 2]. Such calculations have the advantage of being able to test the influence of different electrostatic environments in the vicinity of the pSB, such as counterion charges or interaction with water molecules. We have made some progress towards the TD-DFT and *ab initio* MD investigation of a minimal retinal binding pocket model of PR, containing the pSB, the His-Asp cluster, a Y200 mimic and three water molecules [3]. Intriguingly, this minimal model could recapitulate the first excitation energies of all analog pigments of PR tested, including PR:MMAR. However, the origin of the 740 nm absorbance band and its pH dependence is still an open question. We anticipate that refinement of the model by inclusion of other binding pocket residues will yield a more comprehensive analysis. The computational load of quantum mechanical calculations often limits their applicability to systems containing a few hundred atoms. This quantum mechanical description of the pSB and its nearby residues is often combined with a classical mechanical description of the residual protein in a hybrid approach termed as quantum mechanical/molecular mechanics (QM/MM) [4]. QM/MM is rapidly becoming an established technique, and has already been used to investigate bovine rhodopsin [5], bR [6]and SR-II [7]. The expansion of our minimal retinal binding pocket into a full QM/MM description would provide crucial information about the role of the opsin environment in colour tuning. Furthermore, such an analysis would have strong predictive potential to aid further chromophore or protein modification.

The photoisomerization and photocycle dynamics of the novel analog pigments generated in our study are still largely undisclosed. As detailed in **Chapter 5**, we tested various detergent and membrane environments on the WT and analog pigments, towards the goal of further extensive characterization using spectroscopic methods. PR pigments retain their native quaternary structure and have high thermal stability in the

detergent DDM. On the other hand, GR analog pigments can benefit from subsequent incorporation into a membrane environment. We describe the assembly and stabilization of GR in soluble MSP-nanodiscs, which are too small to contain the PR oligomeric assembly. The optical transparency of both the detergent and nanodisc assemblies enables them to be studied using kinetic spectroscopy. The characterization of the early photo-intermediates of various analog pigments generated in our study is currently underway using femtosecond time-resolved spectroscopy in a DDM and nanodisc microenvironment. Liposomes are another suitable membrane mimic, which are widely used in the ssNMR investigation of various microbial rhodopsins. We have made an initial effort towards the incorporation of PR and GR containing a $^{13}C_{10}$ retinal chromophore into liposomes. We hope to extend this investigation with uniform ^{15}N labeling of the opsin and ^{13}C labeled retinal analogs, such as MMAR, to study the impact of perturbations to the pSB or the binding pocket.

6.2 Further protein or chromophore engineering

In this thesis, we describe the construction of some site-directed mutants described in **Chapters 2 and 3**, which were further combined yielding several double and triple mutants listed in the appendix (Figure A.5). However, most mutation combinations impair the proton-pump function of the pigments, which presents a serious drawback. The combination of individual red-shifted mutations seems to have a limited additive effect on spectral properties and is increasingly deleterious on pumping activity. This is characteristic for the low predictive power of this approach. To overcome such limitations, we describe the development of a novel chemo-taxis assay in **Chapter 4**, which enables the laboratory scale evolution of PR and GR. The highlight of this assay is the simultaneous selection of spectrally shifted variants with conserved or even improved proton-pumping function from a library of random mutations. We anticipate that this method will be useful to select for novel red-shifted opsin mutants.

Furthermore, it can be combined with the retinal analog and mutant combinations discussed in this thesis. For instance, GR-FS:MMAR, which has significant pump activity under NIR illumination, can be used to evolve further red-shifted pigments or the pump activity of PR-DNFS:MMAR can be augmented under high light illumination.

In **Chapters 2 and 3**, we report on modifications at the ring end of the retinal chromophore. It was previously shown by various groups that introducing electron-withdrawing substituents near the polar end of retinal, such as fluoride or trifluoromethyl (CF_3), generate red-shifts in the absorbance bands of bR and bovine visual rhodopsin (Rh)[8]. 13-CF_3 retinal was found to form active red-shifted analog pigments with Rh [9] and bR [10]. We tested the combination of modifications near the ring and polar end of the retinal by introducing a 13-CF_3 substituent in the retinals A2 and MOA2 (see Appendix, Figure A.3). To our surprise, neither of these analogs generated stable analog pigments of PR or GR. The lack of a corresponding stable holo-pigment suggests that the 13-CF_3 derivative does not constructively fit within the retinal binding pocket, possibly because of higher thermal stability of the 13-*cis* isomeric state. On the other hand, 14-F substituted retinal A1 yielded 40-60 nm red-shifted active analog pigments with PR and GR (data not shown), which was also previously reported for bR [8, 11] and Rh [9]. Furthermore, introduction of a 14-F substituent in retinal derivatives with ring modifications generated additional large red-shifts. 14-F MOA2 induced a ~130 nm red-shift in the absorbance of Rh and iodopsin [12], while 14-F azulenic derivatives were shown to cause 170-230 nm red-shifts in the absorbance bands of bR [8]. However, the activity of these pigments is severely attenuated. In fact, the non-substituted azulenic pigments already have very low activity [8, 12]. Nonetheless, introduction of a 14-F substituent in MMAR would be a realistic option to further red-shift of or enhance the low-energy bands in the absorbance profile. Since enamine resonance structures probably

contribute to these low-energy bands of the MMAR pigments (c.f. figure 3.7), another option would be to enhance the electrodonating character of substituents at the aromatic ring without compromising the fit in the binding pocket.

Such red-shifting retinal derivatives have broad application, since in principle they will directly incorporate in all opsin classes, or can be made to fit using small modifications or protein mutagenesis. Examples of some applications are discussed in the following sections.

6.3 Complementing photosynthesis

Photosynthetic microorganisms such as cyanobacteria and algae hold great promise as autotrophic cell factories in the sustainable conversion of solar energy into liquid fuels and biomass [13]. Cyanobacteria in particular are attractive systems to engineer towards the production of important biomolecules or biofuels due to their high growth rates, well-characterized genome and the potential for the direct conversion of energy towards a desired product [14-17]. However, a major bottleneck in the industrial scale application of these microorganisms is that their photosynthetic efficiency is quite low, maximally in the order of 12% [18]. There are several structural and thermodynamic constraints responsible for this low efficiency . The organisms generate only enough ATP to fuel their needs for growth and survival. Furthermore, the net amount of photon energy utilized by photosynthesis is limited. At higher light intensity, structural changes are activated which lead to non-photochemical quenching of the light harvesting complexes, as a photoprotective mechanism. Another important limitation is the spectral range usable by oxygenic photo-synthesis, termed the photosynthetically active radiation (PAR; 400-700 nm), which accounts for less than half the number of solar photons that reach the earth's surface. This is dictated by the absorbance bands of the predominantly exploited chlorophylls a and b (Chl *a* and Chl *b*) in the blue

and red region of the PAR. By exception, some cyanobacteria are able to produce Chl *d*, extending their PAR to about 720 nm. It was postulated that a method of increasing oxygenic photosynthetic efficiency could be accomplished by expanding the wavelength sensitivity of the system to utilize photons outside the PAR [19, 20]. In fact, using photons up to 750 nm would already increase the available photon energy by 19% [19]. An option could be to utilize bacteriochlorophylls, such as BChl *a* and BChl *b*, which are utilized by anoxygenic photosynthetic organisms. Their action spectra extend into the NIR as far as about 1100 nm [21, 22]. However these are relatively complicated systems to engineer into cyanobacteria, and would certainly cause membrane-crowding. Retinal-based phototrophy may be another viable approach towards this end [23, 24].

Rhodopsin proton-pumps have good potential to complement oxygenic photosynthesis due to their light driven function, relatively small genome, ease of engineering, and lack of photobleaching at high light intensities. In the complementary part of our BSC project, we set out to express the proton-pumps PR and GR in the cyanobacterium Synechocystis sp. PCC 6803 and explore their potential to complement oxygenic photosynthesis [24]. The core aim was to express these proton pumps with a batho-chromically shifted absorbance band in the thylakoid membrane in order to allow the transformed organism to utilize photons outside the PAR. Using WT PR and GR pigments, suitable plasmid based expression systems were generated and characterized for *Synechocystis*, and the functional impact of PR and GR expression was assessed [25, 26]. WT PR showed good expression levels and could even stimulate growth of the organism upon illumination, as compared to the non-functional PR D97N mutant, albeit to only a small extent [25]. Assuming that PR-driven energy production could not compete with PSI, it was decided to test a PSI deletion strain of Synechocystis (ΔPSI). Indeed, in this case PR expression gave a significant growth advantage (Chen et al., submitted). The logical follow-up step, viz.

the transformation of the ΔPSI strain with the NIR active PR-DNFS:MMAR described in **Chapter 3** is in progress. We anticipate that despite the lower pump capacity of this analog pigment, supplementation of the resulting strain with NIR light will significantly benefit its growth. This would set a ground-breaking landmark, which after further optimization of such "rainbow strains", would eventually allow to exploit outside-PAR energy input for the microbial production of fuels and commodities.

6.4 Optogenetics

Various light gated ion channels and pumps have found an important application in the field of optogenetics. These channels, broadly classified as cation channelrhodopsins (CCRS) and anion channelrhodopsins (ACRs), are expressed in neurons and are used to selectively depolarize or hyperpolarize the cells upon illumination. This enables precise spatio-temporal control of the activity of neuronal populations and can be even used to modulate their firing frequency. As a consequence, optogenetics has emerged as a very powerful tool in neuroscience and is now widely used to study brain circuitry, or for the pharmacological investigation of specific drugs [27]. The birth of optogenetics was initiated by the discovery of the CCRs Channelrhodopsins1 and 2 (ChR1 and ChR2), which function as light-gated cation channels in the algal eye membrane of *Chlymydomonas reinhardtii* [28, 29]. These channels are transgenically expressed in the neurons of diverse organisms ranging from zebrafish to mammals. They cause neuronal excitation upon illumination with green light, due to an influx of nonspecific cations which cause depolarization of the membrane. On the other hand, the chloride channel Halorhodopsin (NpHR), a member of the ACR family, causes inhibition of neurons by hyperpolarizing the cell membrane upon illumination, due to Cl^- influx [30].

The CCRs and ACRs used in optogenetics have an absorbance maximum between 450-550 nm. Since blue-green light is strongly absorbed and

undergoes significant scattering in biological tissue, most optogenetic techniques in mammalian brain require the implantation of fibre optic cables to deliver light to subcortical tissue. However, the subsequent mechanical perturbation of the tissue may have several non-specific effects, which limits its usability to more superficial brain regions. Thanks to the much lower loss due to scattering, light ≥ 700 nm penetrates much further into biological tissue than blue-green light. Hence, deep-red-light active optogenetic tools are highly desired, and though some effort has been made in this direction [31], NIR activation is still largely inaccessible in this technique.

Chromophore substitution using retinal analogs described in **Chapters 2 and 3** of this thesis provides a complementary strategy to improve the flexibility of these optogenetic tools. Supplementation with MMAR, for instance, would allow improved light penetration and the stimulation of deeper brain regions using NIR illumination. As another example, All-E-6-s-*trans*-locked retinal A2 would enable to red-shift channelrhodopsins, without significant interference with the visual system or signalling metabolites of retinal A1. Multi-wavelength excitation, where distinct neuronal populations are activated or inhibited, is a valuable technique in optogenetics [32], which can be accomplished using red or blue shifting retinal analogs. These analogs can be supplied to the region of interest by stereotactic injection in the tissue, or by dietary supplementation to null mutants in retinal synthesis. Insights obtained from red-shifted mutants generated in the directed evolution assay described in **Chapter 4** will also aid the design of further red-shifted CCRs and ACRs in the future.

6.5 Membrane potential sensors

The non-pumping mutant PR-D97N (PROPS) was recently engineered as a sensor of membrane potential in *E. coli* [33]. While this pigment has almost no pumping activity, it exhibits strong fluorescence, which is sensitive to

the membrane voltage. This was truly a remarkable study, since PROPS facilitates the membrane studies of a small system such as *E. coli*, which is inaccessible to traditional electrophysiological methods. In initial experiments, we saw an at least five-fold enhancement of the fluorescence intensity of PROPS with MMAR relative to A1, with a concomitant red-shift of about 200 nm (Appendix, figure A.7). Such MMAR based voltage indicators would be very useful for the NIR fluorescent imaging of systems, which are too small or difficult to study by conventional means, such as bacteria, neuronal compartments, mitochondria or other cellular organelles.

PROPS could not yet be adapted to eukaryotic cells, due to poor targeting and lack of localization to the plasma membrane. The proton pump AR3 on the other hand shows excellent expression in mammalian neurons, where it was shown to function as an optogenetic neural silencer [34]. Importantly, AR3 also shows fluorescence which is dependent upon the membrane voltage, enabling its potential as a fluorescent sensor in the live-cell imaging of neurons [35]. Such rhodopsin based fluorescent imaging has an advantage over traditionally used fluorescence indicators or calcium imaging, due to its fast response time and low phototoxicity. The dim fluorescence of AR3 could be enhanced in intensity using site-saturation mutagenesis [36] or in combination with merocyanine retinal analogs to engineer a 8.5 fold brighter NIR fluorescent sensor [37]. Our preliminary results have shown that MMAR red-shifts and broadens the absorbance band of AR3 (data not shown), but its effect on intensity and position of the emission band has not yet been investigated. Nonetheless, we can conclude that dual chromophore and protein modification holds great potential in optimizing NIR fluorescent sensors.

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